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An Ectromelia Virus Protein That Interacts with Chemokines through Their Glycosaminoglycan Binding Domain▽

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Poxviruses encode a number of secreted virulence factors that modulate the host immune response. The vaccinia virus A41 protein is an immunomodulatory protein with amino acid sequence similarity to the 35-kDa chemokine binding protein, but the host immune molecules targeted by A41 have not been identified. We report here that the vaccinia virus A41 ortholog encoded by ectromelia virus, a poxvirus pathogen of mice, named E163 in the ectromelia virus Naval strain, is a secreted 31-kDa glycoprotein that selectively binds a limited number of CC and CXC chemokines with high affinity. A detailed characterization of the interaction of ectromelia virus E163 with mutant forms of the chemokines CXCL10 and CXCL12α indicated that E163 binds to the glycosaminoglycan binding site of the chemokines. This suggests that E163 inhibits the interaction of chemokines with glycosaminoglycans and provides a mechanism by which E163 prevents chemokine-induced leukocyte migration to the sites of infection. In addition to interacting with chemokines, E163 can interact with high affinity with glycosaminoglycan molecules, enabling E163 to attach to cell surfaces and to remain in the vicinity of the sites of viral infection. These findings identify E163 as a new chemokine binding protein in poxviruses and provide a molecular mechanism for the immunomodulatory activity previously reported for the vaccinia virus A41 ortholog. The results reported here also suggest that the cell surface and extracellular matrix are important targeting sites for secreted poxvirus immune modulators.

One of the main components of the early host immune response required for efficient clearance of an invading pathogen is the directed infiltration of leukocytes to the site of infection or injury. This component depends on the coordinated action of the chemotactic cytokines known as chemokines (7, 38). Chemokines are a family of proteins secreted in response to diverse stimuli, and they alert different subsets of leukocytes through high-affinity interactions with cell surface G-protein-coupled receptors. They are classified into four subfamilies (CXC, CC, C, and CX_3C) based on the number and arrangement of conserved cysteines located at their N-terminal region. Thus far, 47 chemokines and 19 receptors in humans have been identified (50, 53).

Chemokine presentation is also thought to be influenced by chemokine association with glycosaminoglycans (GAGs) on the surface of cells and within the extracellular matrix (22, 38). GAGs are long, linear, sulfated, and highly charged heterogeneous polysaccharides attached to a protein core. GAGs participate in a variety of important biological processes, such as cell attachment, growth factor and cytokine binding, cell proliferation, migration, morphogenesis, and viral pathogenicity (22). Certain chemokines require interactions with GAGs for their in vivo function (36). The GAG interaction is thought to provide a mechanism for retaining chemokines on cell surfaces and facilitating the formation of chemokine gradients that guide cell migration in the context of their inflammatory, developmental, and homeostatic functions (22).

Large DNA viruses (poxviruses and herpesviruses) have evolved extremely sophisticated mechanisms for evading the immune system. One such strategy is to produce homologs of chemokines and chemokine receptors, as well as nonhomologous chemokine binding proteins (vCKBPs) (1, 20) that interfere with host immune function, reinforcing the role of chemokines in antiviral defense. Secreted vCKBPs have no sequence or structural similarity to known cellular proteins and inhibit chemokine activity through different mechanisms (1). vCKBPs may interact with the receptor binding domain of chemokines, thus blocking binding of the chemokine to cellular receptors and the activation of intracellular signaling. Alternatively, vCKBPs may interact with the GAG binding domain of chemokines, disrupting the appropriate presentation and localization of chemokines.

Most poxviruses encode a 35-kDa M-T1 secreted vCKBP, that competitively binds with high affinity and inhibits CC chemokine interactions with cognate receptors, thus preventing chemokine signaling and chemotaxis (3, 21, 43). The same mechanism is used by the orf virus-encoded vCKBP, an inhibitor of many CC chemokines which also binds with high affinity to lymphotactin, a C chemokine (40). Originally identified as a gamma interferon (IFN-γ) receptor homolog (46), the myx-

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oma virus M-T7 protein has been postulated to interact with low affinity with the heparin binding domain of CXCL8 and probably other chemokines within the CXC, CC, and C families (28). More recently, a novel chemokine binding domain in the cytokine response modifier B (CrmB) protein of variola virus (SECRET domain) has been identified (4), and it binds with high affinity to a reduced set of chemokines. This domain is part of the virus-secreted tumor necrosis factor receptors CrmB and CrmD and is found in three other secreted proteins expressed by some poxviruses. Finally, vCKBPs in alphaherpesvirus (glycoprotein G) (9), betaherpesvirus (p21.5 from human cytomegalovirus) (49), and gammaherpesvirus (the M3 protein from murine gammaherpesvirus 68) (35, 47) have also been described previously.

The vaccinia virus (VACV) A41 protein has sequence similarity to the 35-kDa vCKBP from VACV and other poxviruses. It shares 57% amino acid sequence similarity and 20.5% amino acid sequence identity with the vCKBP of VACV strain Western Reserve. Immunomodulatory activity in different models has been shown for this VACV protein. Ng et al. (34) reported that deletion of the *A41L* gene from VACV strain Western Reserve enhanced virulence slightly and showed an altered inflammatory response to infection in a dermal model. Clark et al. (15) showed that a VACV modified virus Ankara (MVA) strain lacking *A41L* induced better protection than the control virus did. However, none of these studies identify the ligand(s) for A41. In this study, we sought to define the ligand for the E163 protein encoded by ectromelia virus (ECTV) strain Naval, an ortholog of the VACV A41 protein, that shares more than 95% amino acid sequence similarity and 90% amino acid sequence identity with the VACV protein. ECTV is a highly virulent mouse pathogen that causes the disease mousepox in mice. Like variola virus, the causative agent of smallpox in humans, ECTV has a restricted host range, causes a severe generalized disease with a high mortality, and has been used as a model for smallpox (18). Our results demonstrate that E163 is a novel vCKBP that interacts with the GAG binding domain of chemokines.

MATERIALS AND METHODS

Reagents. Recombinant human and murine cytokines were from Peprotech (Rocky Hill, NJ) or R&D Systems (Minneapolis, MN) (39 human and 30 murine chemokines, 15 human tumor necrosis factor superfamily ligands [TNFSFLs]), 2 human IFNs, and 2 murine IFNs were tested). A complete list of the cytokines used is given in Table 1. Biotin-conjugated heparin (from bovine intestinal mucosa) was from Calbiochem. Heparin-agarose beads (cyanogen bromide activated), heparin (from porcine intestinal mucosa), heparan sulfate (from bovine kidney), chondroitin sulfate A (from bovine trachea), chondroitin sulfate B (from porcine intestinal mucosa), and monoclonal anti-His antibody were purchased from Sigma. Activated CH Sepharose 4B beads were from Amersham Biosciences.

Wild-type CXCL12α, CXCL12 3/6 (K24S/H25S/K27S), CXCL12 4-67, and CXCL12 P2G were synthesized by the Merrifield solid-phase method as described previously (5). Recombinant murine CXCL10 purification and mutagenesis to generate and purify the R8A, K26A, R22A, R22E, R20A/R22A, and R22A/K47A CXCL10 mutants was previously described (11).

Cells and viruses. Human monocytes and T cells were grown in 10% fetal calf serum (FCS)-containing RPMI 1640 medium. Hi5 insect cells and *Autographa californica* multiple nucleopolyhedrosis virus were cultured in TC-100 medium supplemented with 10% FCS.

Generation of recombinant baculoviruses. The *E163* gene from ECTV strain Naval (www.poxvirus.org) and the *V003* gene from cowpox virus (CPXV) strain Brighton Red were PCR amplified with specific oligonucleotides, *Pfu* DNA polymerase, and viral DNA as the template. The genes were cloned into pBAC-1 (Novagen) and sequenced. The pBAC-1 recombinant plasmid was cotransfected with linearized baculovirus DNA (BacPAK6; Clontech) into Hi5 insect cells to generate recombinant baculoviruses expressing either the ECTV E163 or the CPXV 35-kDa protein, which was plaque purified in three consecutive steps and amplified as described previously (2).

TABLE 1. Human and murine cytokines tested by SPR as putative binding partners of E163

Cytokine or ligand	Level of E163 binding to indicated ligand type[a]	
	Human	Murine
Chemokines		
CCL1	None	ND
CCL2	None	None
CCL3	None	None
CCL4	None	None
CCL5	Low	None
CCL6	NA	None
CCL7	None	None
CCL8	Low	None
CCL9/10	NA	None
CCL11	None	Low
CCL12	NA	None
CCL13	None	NA
CCL14	None	NA
CCL15	None	NA
CCL16	Low	NA
CCL17	None	None
CCL18	None	NA
CCL19	None	None
CCL20	None	Low
CCL21	High	High
CCL22	Low	None
CCL23	None	NA
CCL24	Low	High
CCL25	High	High
CCL26	High	NA
CCL27	Low	High
CCL28	High	High
CXCL1	None	None
CXCL2	None	None
CXCL3	None	NA
CXCL4	Low	Low
CXCL5	None	None
CXCL6	Low	NA
CXCL7	None	NA
CXCL8 72aa	Low	NA
CXCL8 77aa	None	NA
CXCL9	Medium	Medium
CXCL10	Medium	Medium
CXCL11	Medium	Medium
CXCL12α	High	High
CXCL12β	High	High
CXCL13	Medium	High
CXCL14	High	High
CXCL15	NA	None
CXCL16	None	None
CX3CL1	None	None
XCL1	Low	ND
TNFSFLs		
TNFSFL1	None	
TNFSFL2	None	
TNFSFL4	None	
TNFSFL5	None	
TNFSFL6	None	
TNFSFL8	None	
TNFSFL9	None	
TNFSFL10	Low	
TNFSFL11	None	
TNFSFL12	None	
TNFSFL13	Low	
TNFSFL13b	None	
TNFSFL14	Low	
TNFSFL15	None	
TNFSFL18	None	
IFN		
IFN-α	None	None
IFN-γ	Low	Low

[a] ND, not determined; NA, not commercially available or not yet identified; low, an R_{max} of <100 RU obtained at the end of the injection; medium, an R_{max} of <150 RU and a R_{max}/R at 250 s of <1.5 (this parameter gives the stability for the complex); high, an R_{max} of >150 RU and a R_{max}/R at 250 s of <1.5.

Protein purification. Hi5 insect cells were infected at a high multiplicity of infection with the recombinant baculovirus. Cell supernatant was harvested 72 h postinfection, clarified at 1,000 × *g* for 5 min, and concentrated to 2.5 ml by using Stirred Ultrafiltration Cell 8200 (Amicon). Supernatant was then desalted and buffer exchanged against phosphate buffer containing 10 mM imidazole (PD-10 desalting columns; Amersham Biosciences). The protein (expressed fused to a C-terminal six-His tag) was purified by metal chelate affinity chromatography (Ni-nitrilotriacetic acid resin; QIAGEN). Purified recombinant protein containing fractions were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and then were pooled, concentrated, and dialyzed against phosphate-buffered saline (PBS) by using a Vivaspin500 device (VivaScience).

Chemokine binding to cells. To determine chemokine binding to Molt4 cells, recombinant purified E163 protein was preincubated with 100 pM ^{125}I-CCL25 for 1 h at 4°C. Subsequently, 2.5×10^6 cells were added and incubated for 2 h at 4°C. The amount of bound ^{125}I-labeled chemokine was determined by phthalate oil centrifugation (3).

Chemotaxis assay. ChemoTx 96-well microplates (Neuroprobe, Inc.) were used to determine the ability of recombinant E163 to block chemokine-induced migration of Molt4 or MonoMac cells in an in vitro assay. Chemokine diluted in 0.1% FCS-RPMI 1640 was placed in the lower compartment in the presence of various amounts of recombinant protein. This mixture was preincubated for 15 min at 37°C. Then, 2.5×10^5 cells were placed in the upper chamber, separated by a 3-μm-pore-size filter. After the cells were incubated for 3 h at 37°C, the cells in the lower chamber were counted by incubating the cells for 1 h at 37°C in Cell Titer 96 Aqueous One solution (Promega) and reading the plate at 492 nm.

Heparin-agarose pull-down assay. ECTV E163 protein or CPXV 35-kDa protein (50 nM) was incubated with 10 μl of heparin-agarose beads (50%, vol/vol) in 400 μl of binding buffer (PBS with 0.2% bovine serum albumin) on a rotating wheel for 1 h at room temperature. The beads were then recovered by centrifugation in a microcentrifuge for 1 min at 14,000 rpm. The supernatant was discarded, and the beads were washed three times with 400 μl of PBS. Bound viral protein was eluted from the beads by the addition of 25 μl of SDS-PAGE loading buffer, boiled for 1 min, and then subjected to SDS-PAGE and visualized by immunoblotting.

Characterization of protein-protein interactions by SPR. Cytokine binding specificity and affinity constants were determined by surface plasmon resonance (SPR) by using a BIAcore X biosensor. For ligand screening experiments, purified recombinant E163 protein was amine coupled to a CM5 sensor chip (BIAcore, Inc.) to a level of approximately 4,000 response units (RU) (4,000 pg/mm^2). Recombinant cytokines were injected at 100 nM into HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% [vol/vol] surfactant P20 [pH 7.4]) at a flow rate of 10 μl/min and association and dissociation monitored. The surface was regenerated after each injection by using 10 mM glycine-HCl, pH 2.0. For kinetic analysis, the recombinant protein was immobilized at a low density (R_{max}, <200 RU) to minimize the effects of mass transfer. Different concentrations of the corresponding chemokine were then injected at a flow rate of 30 μl/min over a 2-min period and allowed to dissociate for an additional 5 min. Kinetic data were globally fitted to a 1:1 Langmuir model. All BIAcore sensorgrams were analyzed using BIAevaluation 3.2 software. Bulk refractive index changes were removed by subtracting the reference flow cell responses and the average response to a blank injection was subtracted from all analyte sensorgrams to remove systematic artifacts.

Wild-type and mutant chemokines at 100 nM were injected over the E163-coupled sensor chip at a 10 μl/min flow rate, and the maximum response was recorded. Kinetic parameters were also determined by injecting various concentrations of chemokines over the low-density-protein-coupled chip at a high flow rate under the same conditions described before. Kinetic data were globally fitted to a 1:1 Langmuir model.

Heparin binding analysis by SPR. To measure the kinetic and affinity binding parameters between E163 and heparin, we developed an artificial GAG surface by using a streptavidin (SA) sensor chip (BIAcore, Inc.). The sensor chip was conditioned with three consecutive injections of 1 M NaCl in 50 mM NaOH according to the manufacturer's instructions. The active binding surface was constructed by immobilizing 100 RU of biotinylated heparin onto flow cell 2. For kinetic studies, E163 protein was serially diluted into HBS-EP buffer, and 60 μl of each concentration of protein was injected over both flow cells at a rate of 30 μl/min. Following the association phase, HBS-EP was allowed to flow over both cells to monitor the dissociation phase for up to 5 min. The surface was regenerated by eluting bound protein with a 30-μl injection of 2 M NaCl. Bulk refractive index changes were removed as detailed above, and curves were globally fitted using a 1:1 mass transport binding model.

GAG competition assays using SPR. A GAG competition assay was performed by SPR to measure the relative affinity of E163 for chemokines and heparin. Constant amounts of various chemokines were preincubated with increasing concentrations of soluble heparin. Samples were then injected over the E163-coupled CM5 sensor chip at a 10-μl/min flow rate. The maximum response was measured at the end of the injection.

To examine the oligosaccharide structures that E163 may be capable of binding, we used the heparin-SA sensor chip in an SPR competition assay that involved the preincubation of 100 nM of E163 with several concentrations (0, 1, 10, 100, and 1,000 μg/ml) of soluble GAG competitors, heparin, heparan sulfate, chondroitin sulfate A, and chondroitin sulfate B, for 30 min prior to SPR analysis. The protein preincubated with the corresponding GAG was injected over the chip at a 10-μl/min flow rate, and the response at equilibrium was recorded.

RESULTS

The ECTV E163 protein binds chemokines. VACV A41 was initially described as an immunomodulatory protein with anti-inflammatory properties in vivo (15, 34). However, the host ligand for this protein has not been identified so far. This protein is highly conserved among orthopoxviruses, with more than 80% amino acid identity (Fig. 1A), and is expressed in all tested VACV and CPXV strains (34). ECTV strain Naval contains a gene named *E163* which codes for a VACV A41 ortholog (www.poxvirus.org); this gene is identical to that found in the Moscow strain of ECTV (gene *M140*) (14).

In order to identify the ligand that interacts with ECTV E163, the protein was expressed fused to a C-terminal six-His tag and purified by metal chelate affinity chromatography. Protein fractions were detected by SDS-PAGE and Coomassie blue staining (Fig. 1B). Fractions were pooled and buffer exchanged into PBS, and the sample was subjected to immunoblotting by using an anti-His antibody to confirm that the purified protein was indeed the E163 protein (Fig. 1C). The size of the recombinant protein (31 kDa) suggests that it is a glycoprotein, since the mass of the predicted open reading frame product is 25 kDa and is similar to that described for the VACV A41 ortholog (30 kDa) expressed from VACV-infected cells (29). This recombinant protein was used in further experiments.

The similarity of the ECTV E163 protein to the poxviral 35-kDa M-T1 vCKBP family together with the in vivo results obtained by Ng et al. (34) suggested that E163 may have a similar ability to interact with some proinflammatory host factors, such as chemokines or cytokines, and thus regulate leukocyte infiltration. We therefore sought to identify possible ligands by using SPR (BIAcore X biosensor). We immobilized the recombinant E163 protein at a high density onto a BIAcore CM5 sensor chip. All commercially available human and murine chemokines (69 in total) were tested, and the E163 protein was found to specifically bind some CC and CXC chemokines (Table 1 and Fig. 2), indicating that E163 is a vCKBP. No significant binding was detected to other cytokines, such as IFN-α and IFN-γ (of human and murine origins), or 15 different human TNFSFLs (Table 1).

To assess the affinity of the E163 interaction with chemokines, we performed kinetic binding analysis of the chemokines found to best bind E163. We immobilized E163 at low density (R_{max} of <200 RU) onto a CM5 sensor chip, and chemokines were injected at various concentrations at a high flow rate. As summarized in Table 2, the E163 protein interacted with some

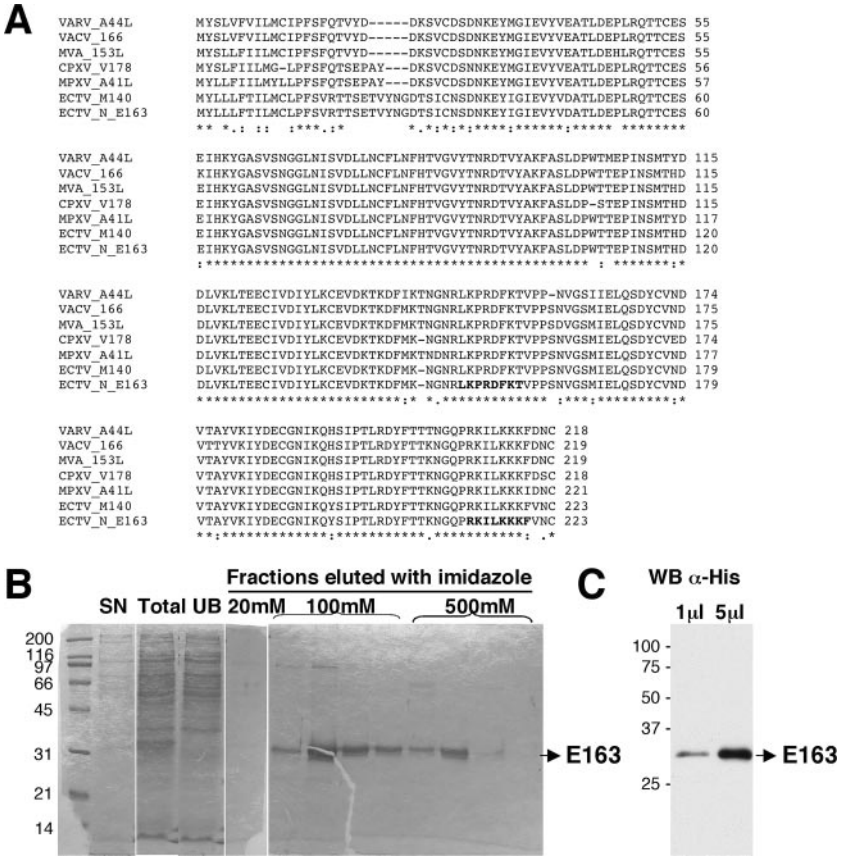


FIG. 1. Expression and purification of the ECTV E163 protein from strain Naval. (A) Alignment (created using CLUSTALW) of the amino acid sequences of the E163 protein orthologs from different orthopoxviruses. Identical (*) and conserved (· and :) residues are indicated. The two putative GAG binding sites in the E163 sequence according to Cardin and Weintraub (12) are indicated in bold. VARV_A44L, variola virus strain Bangladesh (Uniprot/TrEMBL Q85397); VACV_166, VACV strain Western Reserve (Uniprot/TrEMBL Q76ZN6); MVA_153L, VACV strain MVA (Uniprot/TrEMBL O57545); CPXV_V178, CPXV strain Brighton Red (Uniprot/TrEMBL Q8QMR3); MPXV_A41L, monkeypox virus strain Zaire-96-I-16 (Uniprot/TrEMBL Q8V4T8); ECTV_M140, ECTV strain Moscow (Uniprot/TrEMBL A8JL79); ECTV_N_E163, ECTV strain Naval (www.poxvirus.org). The names of the open reading frame products are indicated in the designations. (B) The different steps of the purification of recombinant E163 from supernatants of Hi5 cells infected with a recombinant baculovirus are shown (supernatant [SN] and unbound material [UB]). Protein samples were analyzed by SDS-PAGE and Coomassie blue staining. (C) Purified ECTV E163 protein analyzed by Western blotting (WB) with a monoclonal anti-His antibody (α-His). Molecular size markers (kDa) and the position of the E163 protein are indicated.

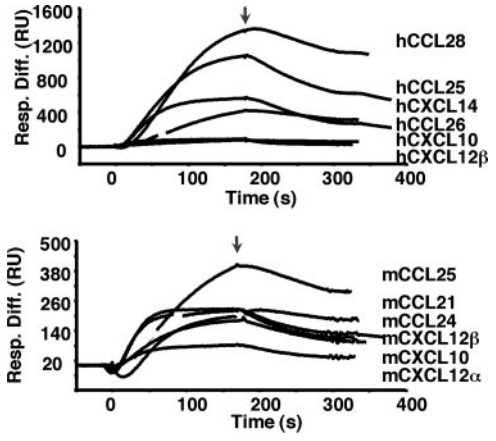


FIG. 2. The E163 protein binds chemokines. Sensorgrams show binding of the indicated human (h) and mouse (m) chemokines to purified recombinant E163, as analyzed by SPR. Arrows indicate the time of the end of the injection, and the times are shown in seconds. Resp. Diff., response difference.

chemokines with high affinity. Representative examples of the fittings generated are available upon request. In contrast to the poxvirus 35-kDa vCKBP, which binds to most members of the CC chemokine subfamily (10), the E163 protein interacts with both CC and CXC chemokines, but it interacts with only a few members of these two subfamilies of chemokines. In fact, the high-affinity binding of E163 to chemokines is restricted to only six CC chemokines (CCL21, CCL24, CCL25, CCL26, CCL27, and CCL28) and three CXC chemokines (CXCL12α, CXCL12β, and CXCL14). There were no significant differences in the calculated K_D (equilibrium dissociation constant) for most chemokines, regardless of their human or mouse origin. It should be noted, however, that human CCL24 and CCL27 did not bind, whereas the murine ligands showed good binding affinity. Also, no murine CCL26 has been identified yet.

Ng et al. (34) found weak binding of VACV A41 to the CXC chemokines CXCL9, CXCL10, and CXCL11. In our experiments, we found that E163 also interacts with those three CXC

TABLE 2. Kinetic parameters and derived affinity constants of the binding of E163 to the indicated human and mouse chemokines

Chemokine[a]	k_a (10^5/M · s)	k_d (10^{-3}/s)	$t_{1/2}$ (s)[b]	K_D (nM)
hCCL21	6.16	7.82	89	12.7
hCCL25	4.08	4.03	172	9.87
hCCL26	4.62	5.26	132	11.4
hCCL28	4.95	0.51	1359	1.04
hCXCL12α	3.59	5.87	118	16.3
hCXCL12β	1.76	2.26	307	12.9
hCXCL14	1.43	2.73	254	19.1
mCCL21	0.73	1.86	373	25.6
mCCL24	2.95	3.04	228	10.3
mCCL25	4.27	7.23	96	16.9
mCCL27	2.45	3.7	187	15.9
mCXCL12α	2.31	5.46	127	23.6
mCXCL12β	2.62	3.95	175	15.1

[a] h, human; m, murine.
[b] $t_{1/2}$, half-life.

chemokines, and we determined the binding affinities for CXCL10 to be 829 nM (k_a [association rate], 0.03×10^5/M · s; k_d [dissociation rate], 2.72×10^{-3}/s) for the human ligand and 556 nM (k_a, 0.07×10^5/M · s; k_d, 4.24×10^{-3}/s) for the murine chemokine. These affinity values are at least 30-fold lower than those determined for the chemokines that bind best to E163.

The affinities calculated for the interaction of E163 with chemokines were within the nanomolar range and similar to the affinity of most chemokines for their receptors (32). This suggested that the E163 protein should be able to interfere with the biological activities of these chemokines, either by competing for the binding to their receptors or by preventing binding to the GAGs present in the membranes of the endothelial cells.

The ECTV E163 vCKBP does not block the interaction of chemokines with specific receptors and the induction of cell migration. To determine whether ECTV E163 inhibits the biological function of chemokines, we first tested the ability of E163 to block chemokine-mediated migration of human monocytes (MonoMac cells) of T cells (Molt4 cells). Purified recombinant E163 was not able to inhibit chemotaxis of Molt4 (Fig. 3) or MonoMac cells (not shown) in response to human CXCL12β (K_D, 12.9 nM) or CCL25 (K_D, 9.87 nM) in spite of their high binding affinity. As a control, purified recombinant ECTV CrmD, a secreted viral protein with a chemokine binding domain and with a K_D of 2.2 nM for murine CCL25 (4), completely abolished cell migration (Fig. 3). These results suggested that E163 does not prevent the binding of these chemokines to their cellular receptors. In accordance with this, no blockade of radioiodinated CCL25 binding to high-affinity Molt4 cellular receptors in the presence of a 250× molar excess of E163 was observed (not shown).

The addition of GAGs interferes with the interaction of the E163 vCKBP with chemokines. Chemokines interact with GAGs such as heparin and heparan sulfate. This interaction is thought to facilitate chemokine localization and does not interfere with chemokines binding to their receptors. To ascertain whether E163 bound to the GAG binding site of chemokines, CCL25, CXCL10, or CXCL12β was preincubated with various doses of heparin before being injected over the E163-coupled sensor chip. Figure 4A shows that heparin effectively competed in a dose-dependent manner with those chemokines for binding to E163, suggesting that the interaction of E163 with chemokines involves the proteoglycan binding domain of chemokines. Heparin competed more efficiently in binding of CXCL10 than in binding of the other two chemokines (at least one order of magnitude better), consistent with the lower affinity of the interaction of this chemokine with E163.

Identification of the E163 binding site in the chemokines CXCL10 and CXCL12α. In order to identify the region of the chemokine that interacts with E163, we tested the ability of E163 to bind, in an SPR assay, to a variety of chemokine analogs of CXCL10 and CXCL12α in which either the receptor or GAG binding site had been mutated.

The regions of CXCL10 involved in the interaction with its cellular receptor CXCR3 and with GAGs have been previously characterized (11). The N-terminal residue Arg-8 of CXCL10 was found to be critical for binding to and signaling through the chemokine receptor CXCR3 without affecting GAG interaction. It was also demonstrated that the GAG binding site of CXCL10 partially overlaps with the CXCR3 binding site. Residues Arg-20, Arg-22, Ile-24, and Lys-26, as well as Lys-46 and Lys-47, were found to constitute the main GAG binding domain, with the mutation of Arg-22 resulting in the largest reduction of heparin binding affinity. Four single mutants (R8A, K26A, R22A, and R22E mutants) and two double mutants (R20A/R22A and R22A/K47A mutants) were tested. The binding RU obtained allowed us to detect significative differences among the distinct CXCL10 derivatives, although no affinity analysis was carried out. As shown in Fig. 4B, the R8A mutant, which does not bind to the receptor but retains the ability to interact with GAGs, bound to E163 at similar levels

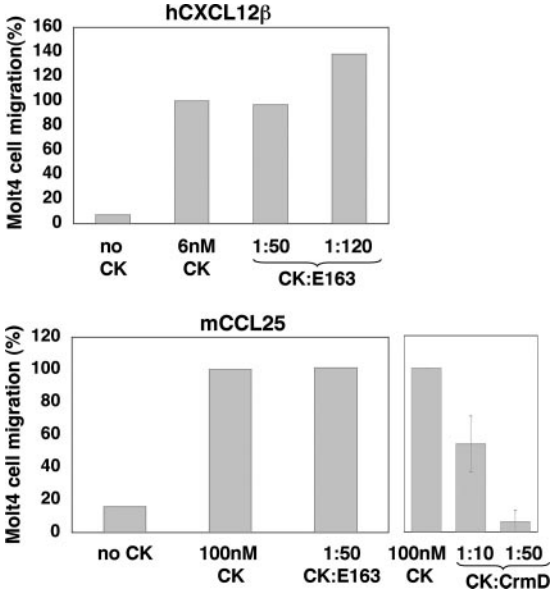
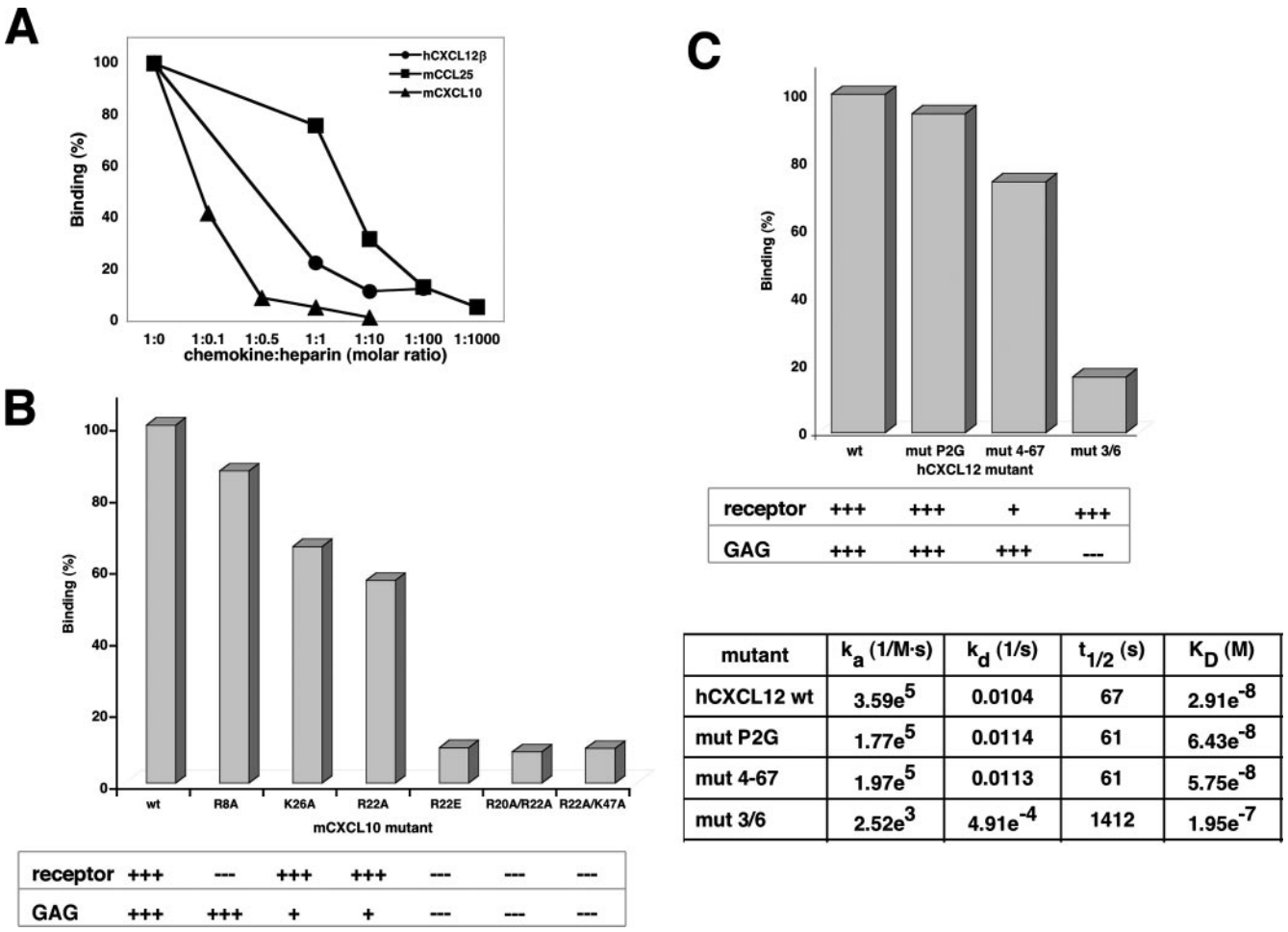


FIG. 3. The E163 protein does not inhibit chemokine (CK)-induced cell migration. Levels of human CXCL12β (hCXCL12β)- and mouse CCL25 (mCCL25)-induced Molt4 cell migration are shown in the absence (100% cell migration) or presence of the indicated molar excess of purified recombinant E163 or control ECTV CrmD. The values (± standard deviations) are the means of two independent experiments.



mutant	k_a (1/M·s)	k_d (1/s)	$t_{1/2}$ (s)	K_D (M)
hCXCL12 wt	$3.59e^5$	0.0104	67	$2.91e^{-8}$
mut P2G	$1.77e^5$	0.0114	61	$6.43e^{-8}$
mut 4-67	$1.97e^5$	0.0113	61	$5.75e^{-8}$
mut 3/6	$2.52e^3$	$4.91e^{-4}$	1412	$1.95e^{-7}$

FIG. 4. The interaction between E163 and chemokines involves the GAG binding domain of the chemokines. (A) SPR binding assay for mouse (m) CCL25 or CXCL10 or human (h) CXCL12β to purified E163 in the presence of increasing concentrations of heparin. Chemokine and heparin were incubated for 15 min before injection over an E163-coupled chip, and the maximum response was recorded. The percentage of binding refers to binding in the absence of heparin. (B) Binding of murine (m) wild-type CXCL10 (wt) (100%) and CXCL10 analogs to purified E163 by SPR. The ability of the CXCL10 analogs to interact with cellular receptors or GAGs is indicated. A 100 nM injection of each analog over the E163-coupled chip was performed, and the response at equilibrium was recorded. The values are the means of two independent experiments. (C) Binding of 100 nM wild-type CXCL12α (wt) (100%) and CXCL12α mutants (mut) to purified E163 by SPR. The abilities of the CXCL12α analogs to interact with cellular receptors or GAGs are indicated. A 100 nM injection of each analog was performed, and the response at equilibrium was recorded. The values are the means of three independent experiments. Various concentrations of the analogs were injected at a high flow rate, and kinetic data were globally fitted to a 1:1 Langmuir model. Derived affinity constants are shown in the tables. $t_{1/2}$, half-life. hCXCL12, human CXCL12. The binding of the chemokine to the receptor or GAGs is indicated: +++, high binding; +, low binding; −, no binding.

as those of the wild-type CXCL10. By contrast, the rest of the analogs, which have mutations that affect the GAG binding site, showed a reduced ability to interact with E163. This outcome suggested that E163 interacts with this chemokine through its GAG binding domain, as analogs which have disruptions in the GAG binding domain are unable to bind to E163.

The regions of CXCL12α involved in receptor and GAG binding have also been well characterized. The N-terminal four residues of CXCL12α (Lys-1, Pro-2, Val-3, and Ser-4) are essential for receptor binding and activation (16), whereas the Lys-24, His-25, and Lys-27 cluster of residues constitutes an essential component of the heparin binding site, which in turn is distinct from that required for binding to and signaling through the receptor (5). Residue Lys-1 of the chemokine is also involved in GAG binding. A similar set of experiments performed with CXCL10 derivatives was carried out with analogs of CXCL12α in which either the receptor or GAG binding site was abolished. As shown in Fig. 4C, wild-type CXCL12α and a protein with a mutation of the Pro-2 residue (the P2G mutant of CXCL12α), which has been shown to be essential for receptor activation, bound to E163 at the same level. The CXCL12α 4-67 mutant, which lacks the first four residues needed for receptor binding, bound to E163 (75% binding relative to wild-type CXCL12α). Finally, the CXCL12α 3/6 mutant (a derivative that has the GAG binding cluster residues Lys-24, His-25, and Lys-27 changed to Ser and that is unable to interact with heparin) showed very low binding to E163. The affinities of the interaction of the four CXCL12α analogs were determined, and they were in accordance with the binding levels

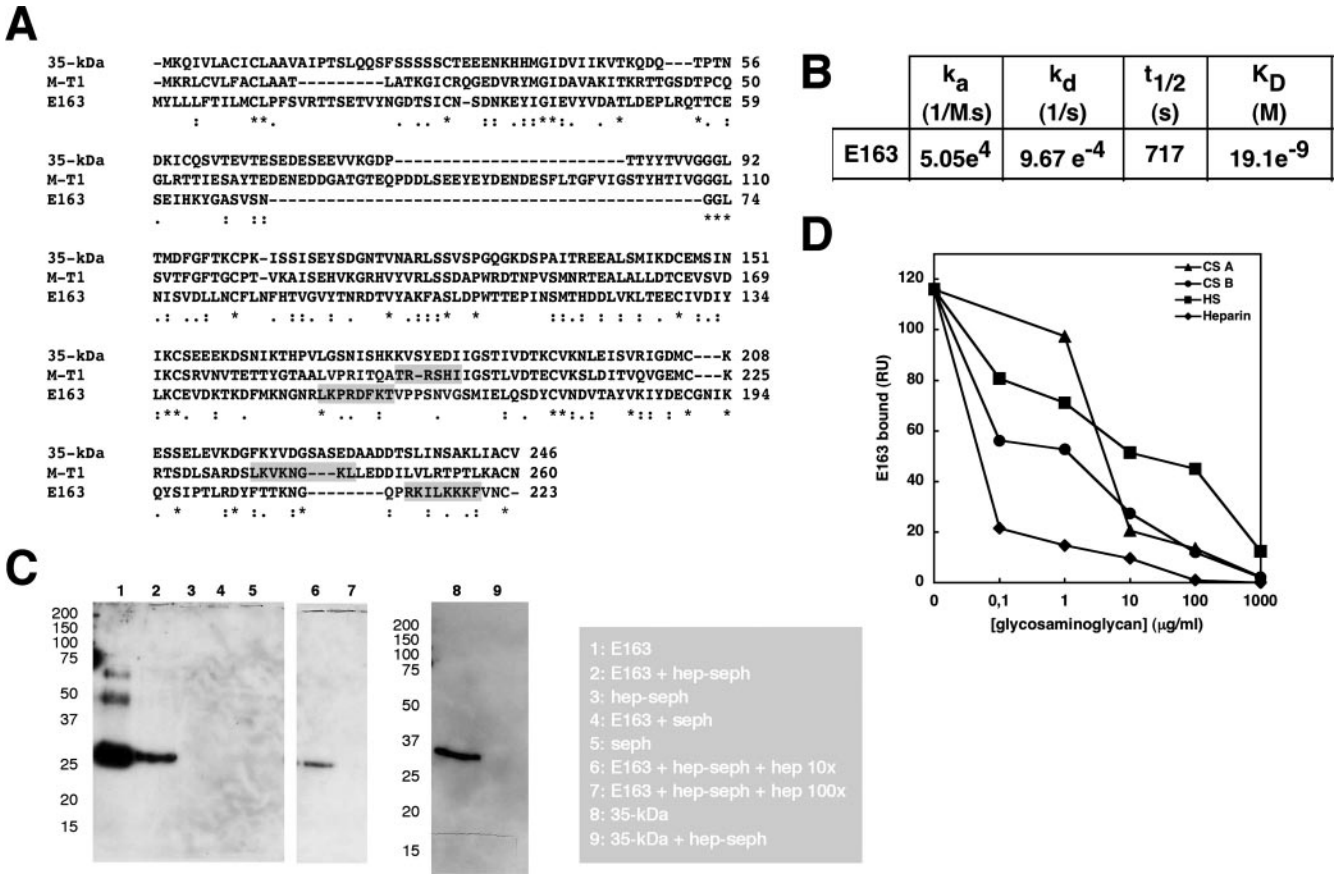


FIG. 5. The E163 protein is a GAG binding protein. (A) Alignment of the amino acid sequences of the CPXV 35-kDa protein (strain Brighton Red; Uniprot/TrEMBL, Q85307), the myxoma virus protein M-T1 (strain Lausanne; Uniprot/TrEMBL, O10625) and the ECTV E163 (strain Naval) (www.poxvirus.org) created using CLUSTALW. Identical (*) and conserved (· and :) residues are indicated. The two putative GAG binding sites in the M-T1 and E163 sequences are highlighted. (B) Kinetic parameters and derived affinity constants of the binding of E163 to an artificial GAG surface. Kinetic data were globally fitted to a 1:1 mass transport binding model. $t_{1/2}$, half-life. (C) Heparin pull-down assay. Lanes 1 (ECTV E163) and 8 (CPXV 35-kDa) show starting material. Purified protein (50 nM) bound to Sepharose (control, lane 4) or heparin-Sepharose (hep-seph) beads (lanes 2 and 9). Lanes 3 and 5 show heparin-Sepharose and Sepharose beads alone, respectively, without protein. Excess soluble heparin was used in lane 6 (10× molar excess) and lane 7 (100× molar excess) to compete the interaction of viral protein bound to the heparin-Sepharose beads. Proteins were subjected to SDS-PAGE and blotted using a monoclonal anti-His antibody. (D) ECTV E163 interacts with other sulfated GAGs. Purified recombinant E163 protein (100 nM) alone or preincubated for 30 min with increasing concentrations of soluble heparin, heparan sulfate (HS), chondroitin sulfate A (CS A), and chondroitin sulfate B (CS B) was injected over a BIAcore SA sensor chip with immobilized biotinylated heparin. The SPR signal was recorded at equilibrium and plotted as a function of the concentration of soluble GAG competitor.

detected. Wild-type CXCL12α as well as mutants with mutations affecting receptor activation and signaling bound with the same high affinity to E163, whereas the CXCL12α 3/6 mutant showed a much lower affinity for E163. This lower affinity was mainly due to a lower association rate, but the complex had a longer half-life. Altogether, these results indicated that E163 interacts with chemokines through their GAG binding site.

The E163 vCKBP is also a GAG binding protein. Analysis of the E163 protein sequence (Fig. 5A) revealed two potential GAG binding regions, ^{152}LKPRDFKT^{159} and ^{213}RKILKKKF^{220}, closely fitting consensus sequence XBBXBX or XBBBXXBX, where B is a basic residue and X is any hydropathic residue (12). Interestingly, the CPXV 35-kDa protein, which does not bind heparin (41), does not possess these motifs (Fig. 5A). Moreover, the myxoma virus M-T1 protein has been shown to interact with GAGs and contains two GAG binding motifs very close to those present in E163, and these have been proposed to be the heparin binding sites for that M-T1 protein (Fig. 5A) (41).

Since several poxvirus proteins have been shown to bind GAGs, we tested by SPR the potential interaction of E163 with heparin. The protein was injected over a BIAcore SA chip coated with immobilized biotinylated heparin, and we found that, consistent with the presence of the GAG binding motifs, E163 interacted with the heparin surface. The affinities and kinetics of the binding were determined by injecting various concentrations of E163 over the heparin surface. Interestingly, E163 bound heparin with a high affinity of 19 nM, comparable to that of chemokines (Fig. 5B).

In order to further characterize the interaction of E163 with GAG, we performed a heparin-Sepharose bead pull-down assay. The E163 protein was incubated with the heparin beads, and bound protein was collected by centrifugation, eluted in SDS-PAGE loading buffer, and immunoblotted using a specific

monoclonal antibody to the His tag. As shown in Fig. 5C, purified E163 bound to heparin-Sepharose, whereas purified 35-kDa vCKBP encoded by CPXV did not bind, as expected. The specificity of the E163-heparin-Sepharose interaction was demonstrated by competitive inhibition in the presence of soluble heparin.

Heparin is commonly used as a generic GAG, although it is the least common component of the extracellular matrix. To demonstrate that E163 can interact with other GAGs, a competition assay was using SPR performed (Fig. 5D). E163 was incubated with various concentrations of soluble heparin, heparan sulfate, chondroitin sulfate A, or chondroitin sulfate B or without GAG and then injected over a BIAcore SA chip coated with immobilized biotinylated heparin. Competitive inhibition by the soluble GAG was detected as a drop in the maximum binding compared with the binding of E163 alone. All soluble GAGs competed with immobilized heparin in a concentration-dependent manner but with different efficacies. Heparin was the most effective competitor, inhibiting the interaction at concentrations lower than 1 μg/ml, whereas the less-sulfated chondroitin sulfates A and B were less potent by at least one order of magnitude. Heparan sulfate exhibited a further 10-fold decrease in the ability to inhibit E163 binding to the heparin surface. These results demonstrated that E163 is capable of interacting with a variety of sulfated GAG structures but with various affinities.

DISCUSSION

Chemokines are crucial mediators of the immune response, and their role in viral infection is underscored by the many strategies that viruses have evolved to modulate their activities and thereby circumvent proinflammatory signals that promote leukocyte infiltration into virus-infected tissues. In the present study, we have characterized the ECTV E163 protein and have identified several chemokines as the binding partners for this secreted viral protein. We have also shown that E163 interacts with GAGs.

The genomes of all orthopoxviruses that have been sequenced contain a gene very similar to *E163*, suggesting that the encoded protein might have an important function. Moreover, the role of the VACV A41 ortholog in vivo has been demonstrated in various models of infection. Ng et al. (34) showed that deletion of the *A41L* gene from VACV strain Western Reserve enhances virulence slightly in a murine intranasal model of infection, and mice exhibited larger lesions with a greater influx of inflammatory cells than control viruses, and the rate of virus clearance was accelerated. Also, the deletion of the *A41L* gene from VACV MVA enhances the antiviral $CD8^+$ T-cell memory response, thus improving vaccine efficacy (15).

The data presented in this paper show that E163 is a novel secreted vCKBP. Interestingly, both ECTV E163 and the 35-kDa protein from VACV or CPXV are secreted viral immunomodulators that bind chemokines with high affinity. However, despite their sequence similarity, their mechanisms of action are different. The 35-kDa vCKBP binds with high affinity most chemokines of the CC subfamily and competes for binding to their cellular receptor, thus inhibiting the biological actions of chemokines (3, 10, 43). ECTV E163 binds chemokines belonging to both CC and CXC subfamilies, although the high-affinity interaction ($K_D < 20$ nM) of E163 with chemokines is limited to 10 chemokines. These chemokines are likely to be relevant for antiviral response, as (i) they mediate T- and B-cell recruitment and are expressed by epithelial cells in mucosal surfaces (CCL25 and CCL28), the skin (CCL27) (23, 26, 29), or the endothelium of afferent lymphatics (CCL21) (45); (ii) CCL24 and CCL26 recruit eosinophils to airway epithelia (25, 42); and (iii) CXCL14 is involved in dendritic-cell migration to epidermal tissues (39). These chemokines were not previously tested by Ng et al. (34) as putative ligands for VACV A41. Indeed, they found weak binding for some chemokines (CXCL9, CXCL10, and CXCL11), although the A41 protein did not inhibit the chemotactic activity of those chemokines in biological assays. We have also detected positive binding for those three chemokines to E163; however, we found that the affinity for CXCL10 was at least 30 times lower than that for the other chemokines.

In contrast to chemokine interaction with the 35-kDa vCKBP, the interaction of E163 with chemokines appears not to involve their receptor binding domain. This concept is supported by several lines of evidence: (i) no inhibition of cell migration in the presence of E163 was observed with CXCL12 or CCL25; (ii) E163 does not inhibit the binding of CCL25 to cells; (iii) heparin effectively acts as a specific inhibitor, displacing the binding of chemokines to E163; and (iv) binding assays for CXCL10 and CXCL12α analogs that have altered either the receptor or GAG binding domains showed reduced binding affinity for those molecules that cannot bind to heparin. We propose, therefore, that the binding of E163 to chemokines may be facilitated through the heparin binding region found in multiple chemokines.

The E163 protein does not inhibit the biological activity of chemokines in vitro in a migration assay. However, the high-affinity interaction of E163 with the GAG binding domain of chemokines is likely to modulate chemokine activity in vivo. The interaction of chemokines with GAGs is thought to be crucial for the correct function of the chemokine network in vivo (36). High-affinity chemokine binding to cell surface GAGs is thought to permit chemokine retention on the luminal surface of endothelial cells under flow conditions and serve in the formation of a concentration gradient leading back to the site of chemokine production (27). These interactions may provide another level of specificity and regulate the types of chemokines immobilized at inflammatory sites, because the contents and compositions of cell surface GAGs differ with the type and location of endothelial tissues (27). In addition, GAG-chemokine complexes may also activate specific signal transduction processes independent of the chemokine receptor interaction or may prevent inappropriate chemokine activation. Such chemokine-GAG complexes may also serve as storage forms for rapid mobilization of chemokines (48). Therefore, the blockade of chemokine-GAG interaction by E163 may modulate the immune response and have beneficial consequences for the virus, as previously demonstrated in models of VACV infection (15, 34).

The absence of mammalian soluble chemokine receptors together with the crucial role that chemokines have in the immune response makes vCKBPs very attractive as therapeutic agents in chemokine-related diseases, and their potential has

been demonstrated in animal models of inflammatory diseases (19, 30). The myxoma virus protein M-T7, 35-kDa poxviral vCKBP, or murine gammaherpesvirus 68 vCKBP M3 immunomodulatory activity has been demonstrated in models of chronic transplant vasculopathy and airway or skin inflammation (8, 19, 24, 30, 31, 37). Different strategies that interfere with chemokine-GAG interactions for therapeutic benefit may be explored. For example, an anti-inflammatory effect of heparin and heparin derivatives has been demonstrated (for a review, see reference 22). Similarly, viral proteins like E163 that specifically block the interaction of some chemokines with GAGs may have therapeutic value.

Our results also show that the E163 protein interact with GAGs in vitro with an affinity (K_D, 19 nM) comparable to that for chemokines. This high affinity is also similar to that calculated for the interaction of several chemokines with GAGs, ranging from 7 nM for CXCL4 to 38.4 nM for CXCL12 (22). Several other immunomodulatory poxvirus proteins have been shown to interact with cell surface GAG molecules, such as the complement control protein from VACV (33, 44), the myxoma virus vCKBP M-T1 (K_D, 446 nM) (41), the variola virus interleukin-18 binding protein D7 (K_D, 55 $\pm$ 17 nM) (17), and the molluscum contagiosum virus interleukin-18 binding protein MC54 (K_D, 0.52 nM) (51). It is likely that this GAG binding activity of several viral secreted immunomodulatory proteins is important in preventing diffusion in vivo and enabling vCKBPs to increase their local concentration at sites of infection. Finally, like other heparin binding proteins, GAG binding by vCKBP may protect against protease degradation. The results presented here for ECTV E163 provide additional support for the view that the cell surface and extracellular matrix are important targeting sites for virus immunomodulatory proteins.

The structures of the 35-kDa proteins from CPXV (13) and ECTV (6) have been determined, and more recently, the complex with a chemokine has been resolved for the rabbitpox 35-kDa protein and CCL4 (52). As predicted, a negatively charged β-sheet was shown to be the main binding surface for chemokines. By use of structural folding models and deletion analysis of M-T1, the heparin binding domain for this protein was localized on a high positively charged region on the opposite face of the molecule (41). The 35-kDa protein, which was shown not to bind heparin, had neither this high positively charged region nor any obvious heparin binding consensus motifs within this region. In the case of ECTV E163, structural folding models (Swiss pdbviewer) reveal an overall structure very similar to that of the 35-kDa protein, with the two predicted heparin binding motifs localized also on the same face of the molecule as that for M-T1, which could thus constitute the heparin binding site on the molecule. Interestingly, many of the chemokines that bind to E163 have been shown to interact also with the SECRET domain, a chemokine binding domain that like E163 binds to a reduced set of chemokines (4). The region in the 35-kDa protein that constitutes the chemokine binding domain involves a large loop that is absent in E163. The transfer of this region to the VACV A41 protein did not confer to A41 the ability to bind chemokines (34). Maybe there is a common structural determinant between the SECRET domain and E163 that confers to these two proteins such a high chemokine specificity.

The work presented here defines ECTV E163 as a new vCKBP encoded by poxviruses. The chemokine family is complex, and we are still learning the biological functions of the different chemokines. Poxviruses may encode simultaneously several vCKBPs. For example, ECTV encodes E163, the 35-kDa protein, and three secreted proteins containing the SECRET domain (CrmD, E12, and E184). It is likely that the function of the vCKBPs is not redundant and their diversity reflects the optimization of the different vCKBPs to efficiently block specific chemokines at different times and sites of infection in the animal host.

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